Blood serum protein profiles and lysozyme activity in dogs during experimental infection with *Staphylococcus intermedius*

T. M. GEORGIEVA1*, M. J. ANDONOVA2, E. P. SLAVOV2, P. V. DZHELEBOV2, D. S. ZAPRYANOVA1, I. P. GEORGIEV1

1Department of Pharmacology, Animal Physiology and Physiological Chemistry; Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, BULGARIA.
2Department of General and Clinical Pathology, Section of Functional Pathology and Immunology, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, BULGARIA.

*Corresponding author: teodoramirchevag@abv.bg

SUMMARY

The present study was conducted in order to evaluate changes in blood serum protein profiles and lysozyme activity in dogs experimentally infected with *Staphylococcus intermedius*. The bacterial suspension (5x10^9 cfu) was subcutaneously injected to 6 healthy adult dogs whereas 6 other healthy adult dogs served as controls and the serum concentrations of total proteins, albumin, globulins, immunoglobulins, lysozyme and the albumin/globulins (A/G) ratios were determined on all dogs for 14 days. Whereas in controls, these parameters remained relatively stable, in infected dogs, proteinemia, albuminemia and A/G ratios have slowly declined and reached minimal values (significantly lower than initial and control values for albuminemia and the A/G ratios) on day 7, whereas the globulin concentrations tended to increase, but not significantly, on days 7 and 14 as the immunoglobulin concentrations. The lysozyme activities were markedly elevated compared to controls since the 2nd after inoculation, were maximal on day 3 and remained significantly higher than the control and the baseline values until the 14th day. These results show that staphylococcal infection has altered the serum protein profiles, mainly in the albuminemia and in the A/G ratio and that the serum lysozyme activity may be considered as the most sensitive marker of this infection.

Keywords: Dog, *Staphylococcus intermedius*, experimental infection, proteinemia, albuminemia, globulins, immunoglobulins, lysozyme.

Introduction

There are well over 200 plasma proteins described and quantified in man and animals, many of which change markedly in disease and many of these changes are quite subtly [14]. These proteins exert physiological functions (transport, humoral immunity, maintenance of oncotic pressure, enzymes, protease inhibitors, buffering) in the plasma, but the functions of a lot of plasma characterized proteins remain to be determined [9].

Classification of plasma proteins is based on electrophoretic mobility: according to it, albumin (Alb) and prealbumin (may not exist in some domestic animals) faster migrate towards the anode, then the a1-globulins (α1-antitrypsin, α1-acid glycoprotein), the α2-globulins (haptoglobin, α2-macroglobulin, ceruloplasmin), the β-globulins (transferrin, low density lipoproteins, C3, C4, haemopexin, C-reactive protein and finally the γ-globulins (fibrinogen produced by the liver and involved in the coagulation pathway [10] and IgM following by IgG, IgA, IgD and IgE) are successively oriented towards the cathode [2, 14]. Albumin is the most prominent of the plasma proteins in animals. It constitutes between 35 and 50% of the total circulating proteins [14]. Its tertiary structure is globoid or ellipsoid, and it is the most homogenous fraction. Albumin is synthesized by the liver, as are all plasma proteins except immunoglobulins, and is degraded by all metabolically active tissues. Albumin is a large, osmotically active protein with an average molecular weight of 69 kDa and a half-life of 8.2 days in dogs [2] which is a major storage reservoir and transporter for amino-acids [2]. Globulins have molecular weights ranging from 90 to 156 kDa (γ-globulins). The immunoglobulins or antibodies are glycoproteins produced by the plasma cells as part

RÉSUMÉ

Cette étude a été conduite dans le but de détecter des modifications dans les profils des protéines sériques et dans l’activité sérique du lysozyme chez des chiens infectés expérimentalement par *Staphylococcus intermedius*. Six chiens adultes en bonne santé ont reçu par voie sous-cutanée une suspension bactérienne (5x10^9 cfu) et 6 autres ont servi de contrôles, puis les concentrations sériques des protéines totales, de l’albumine, des globulines, des immunoglobulines, du lysozyme et les rapports albumine / globulines (A/G) ont été déterminés sur tous les chiens pendant 14 jours. Alors que chez les contrôles, ces paramètres restent relativement stables, la protéinémie, l’albuminémie et le rapport A/G ont lentement diminué chez les chiens infectés pour atteindre des valeurs minimales (significativement plus faibles que les valeurs initiales et contrôles dans le cas de l’albuminémie et du rapport A/G) le 7ème jour, alors que les concentrations des globulines ont tendu à augmenter les 7ème et 14ème jours, comme celles d’immunoglobulines. Les activités du lysozyme ont été considérablement élevées par comparaison aux contrôles dès le 2ème jour après l’inoculation, maximales le 3ème jour et elles sont restées significativement plus fortes que les valeurs contrôles et les valeurs initiales jusqu’au 14ème jour. Ces résultats montrent que l’infection staphylocooccique a altéré le profil des protéines sériques, et principalement l’albuminémie et le rapport A/G et que le lysozyme peut être considéré comme le marqueur le plus sensible de cette infection.

Mots clés : Chien, infection expérimentale, protéinémie, albuminémie, globulines, immunoglobulines, lysozyme.

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of the immune response, whose basic structure is a monomer made up of two heavy and two light chains linked together by disulfide bonds [9].

The biochemistry laboratory tests routinely measure “total protein” and “albumin” concentrations, usually in a serum specimen, and report the “globulin” fraction as the difference between “total protein” and “albumin”. Other proteins (e.g. immunoglobulins) are measured as classes. A decreased total proteinemia usually means that the albumin concentration is low [9]. Changes in serum protein concentrations result in a variety of clinical signs and systemic effects and are associated with a number of disease processes and synthesis of approximately 40 acute phase proteins (APPs) [10]. The concentrations of these plasma proteins often increase (positive APPs) and more rarely decrease (negative APPs) [25] during the acute phase response to inflammation or infection, and play important defensive roles and their expression represents effector mechanisms of innate immunity. These remarkable APPs have been extensively studied in humans and in laboratory animals [3, 10, 23] whereas limited data concerning lysozyme activity were available in the literature.

The present study was conducted to evaluate changes in the concentration of total protein (TP), major plasma proteins – albumin (Alb), globulin (Glb), albumin/globulin (A/G ratio), immunoglobulins (IMG) and lysozyme (Lys) as part of innate immune system during experimental Staphylococcus infection in dogs. This infection was chosen because Staphylococcus intermedius can infect various animal species such as horses, dogs, cats and pigeons [7]. It is established that the bacteria is responsible for around 100% of pyodermitis that is the most described skin disease in these species [4].

Material and Methods

EXPERIMENTAL ANIMALS AND PROTOCOL DESIGN

The experiments were carried out on 12 mail dogs, 4-7 years old, which were individually housed at room temperature (20-22°C) in disinfected metal cages with a slat floor and exposed to a 12 hour light-dark cycle. They were fed with a commercially available diet of dog pellet twice daily “Jambo dog”, (Gallisman S.A., Bulgaria) and had free access to water. Every day, they walked for 30 minutes, twice daily. Ten days before experiment they were treated with Prazimec – D (Biovet, Peshtera, Bulgaria) at dose rate 1 tablet per 10 kg weight and were washed with anti-parasitic shampoo and treated with Ectomin and Tapilan (Dorvet, Israel) against ectoparasites.

Dogs were randomly divided into experimental and control groups: in the experimental group, animals were infected with a 24 hours broth culture of Staphylococcus intermedius (density: 1x10^9 cfu/mL) by subcutaneous injection (5 mL). The strain of Staphylococcus intermedius used in the experiment was isolated from uroculture from a clinical affected dog and serotyped at the Department of Microbiology, Epidemiic and Parasitic Diseases using semi-automated system for the identification BD BBL Crystal Gram Positive ID System and in particular this strain was catalase positive, oxidase negative, coagulase positive and DNAse positive.

Blood samples from each dog were collected by puncture of the v. cephalica anterior into sterile tubes without anticoagulant immediately before infection (hour 0) and 3, 24, 48 and 72 hours as well as 7 and 14 days after the infection challenge. Tubes were kept at room temperature for 2 hours to allow clotting, then centrifuged (1500g, 15 minutes, room temperature) and serum was decanted, carefully harvested and stored at -20°C until assayed. The experimental protocol was approved by the Ethic Committee of the Veterinary Medicine, Stara Zagora.

ANALYTICAL METHODS

Serum concentrations of total proteins, albumin and immunoglobulins were measured in each dog. The total protein concentration was determined by the Biuret method [16] and albuminemia was determined using kit with Bromocrezol green (Gesellschaft für Biochemica, Germany SU-ALBU INF 156001F, Germany) whereas globulins were determined by subtracting the albumin concentration from the total protein concentration. Immunoglobulins were determined by the zinc-sulphate turbidity test [22]. Lysozyme concentration was measured by the method of Lie and Syed [18] on the basis of radial immunodiffusion in agarose gel. Briefly, 20 mL of 2% agarose (ICN,UK, Lot 2050) dissolved in phosphate buffer (0.07M Na2HPO4 and NaH2PO4, pH 6.2) was mixed with a 20 mL suspension of a 24 hour culture of Micrococcus lysodecticus at 67°C. This mixture was poured into a Petri dish (140 mm in diameter). After solidifying at room temperature, 32 wells were made (5 mm diameter) and 50 μL undiluted sera were poured into each well. Eight standard dilutions (from 0.025-3.125 mg/L) of lysozyme (Veterinary Research Institute, Veliko Tarnovo) were added to each well. The samples were incubated for 20 hours at 37°C and lytic diameters were measured.

STATISTICAL ANALYSIS

The statistical analysis of the data was performed using one way analysis of variance (ANOVA). The significance of differences of means between and within groups was evaluated by LSD test and differences were considered as significant when P values were less than 0.05. All data are expressed as mean ± standard deviation (SD).

Results

The presence of infection and disease was assessed on the basis of clinical examination and laboratory findings.

Firstly, in the first 24 hours after the infection, the dogs were lethargic, did not eat or drink. Afterwards, they began gradually to increase food and water intake and on the day 7 the level of consumption was similar to the pre-infection one.

Alterations in the rectal body temperature became evident on the 6th hour post-inoculation, were maximal between the 6th and the 24th hours and persisted elevated until the 48th hour compared to the baseline values. Thereafter, the body temperature in the infected group slowly declined. Surprisingly, the
clinical parameter in not infected animals has also gradually increased according to time, but not significantly compared to the baseline values.

As early as the 24th hour after the inoculation, a restricted, temperate hyperaemic painful swelling has appeared at the injection site. Fourteen days after the infection, at the site of bacterial suspension application in some dogs a purulent yellowish-gray exudate was observed after the fistulisation of the underlying abscess. The adjacent hair coat was stained by the spontaneous discharge.

Bacteriologically, *S. aureus* with the characteristics of the challenging strain was isolated from all swab abscess samples. No *S. aureus* was isolated from the visceral organs.

The global profiles of plasma proteins (concentrations of total proteins, albumin, globulins and the albumin/globulins ratios) in *Staphylococcus* infected dogs compared to the healthy controls were reported in Table I. Whereas the proteinemia remained stable in the control group, this parameter gradually declined in the infected group and reached minimal value on day 7 (decrease percentage: -7.2%) then slightly increased on day 14 but differences with the initial mean value or with control values recorded at the same time points were not statistically significant. No significant changes in albuminemia and in globulin concentrations were detected in control group. By contrast, the staphylococcal infection also caused a progressive and marked fall in serum albumin concentrations on days 3 and 7 after bacterial inoculation compared to the baseline values (*P* < 0.01 on day 3, *P* < 0.001 in day 7, the decrease percentages were -8.5% and -25.5%, respectively). On day 7, the mean serum albumin concentration in experimental group was significantly lower than in the control group (*P* < 0.05). In addition, the globulin fractions tended to increase compared to the initial values 7-14 days after (the increase percentages were 10.7% and 6.8%, respectively). However, differences with the initial and control values were not statistically significant. In groups, the albumin/globulins (A/G) ratio have not significantly changed according to time, although this parameter has greatly fluctuated in *Staphylococcus* infected dogs (from 0.66 ± 0.37 to 1.03 ± 0.50). However, the A/G ratio was significantly lowered on days 7 and 14 compared to the initial values 7-14 days after (the increase percentages were 153.3%, *P* < 0.01) then slowly declined but remained significantly elevated (the increase percentages were 111.7% and 115.0% on days 7 and 14 respectively, *P* < 0.05).

As presented in Table II, the immunoglobulin concentrations slightly increased on day 7 in dogs with experimental staphylococcal infection, but not significantly compared to the pre-infection and control values. On the other hand, the serum lysozyme activities tended to be higher in infected dogs over the whole experimental period than in controls in which they were remarkably stable and low. Differences with controls were highly significant on the days 2 (*P* < 0.05) 3 (*P* < 0.001), 7 and 14 (*P* < 0.01). Compared to the initial values, the lysozyme activity slightly increased 48 hours after *Staphylococcus* inoculation, reached maximal values at 72 hours (the variation percentage was 153.3%, *P* < 0.01) then slowly declined but remained significantly elevated (the increase percentages were 111.7% and 115.0% on days 7 and 14 respectively, *P* < 0.05).

Serum protein profiles were altered in dogs inoculated with *S. intermedius*. The results of the present study revealed that albuminemia, albumin/globulin ratio and especially lysozyme activity (for which changes were greater than those observed for the other parameters) were sensitive factors following *Staphylococcus* infection.

### Discussion

The results of the present study further clarified the behaviour of major blood proteins in response to *Staphylococcus* infection in dogs. It was observed that the serum protein profiles were slightly affected by the experimental staphylococcal

### Table I: Variations of the plasma protein fractions according to time in control healthy dogs (*n* = 6) and in dogs subcutaneously inoculated with a *Staphylococcus intermedius* suspension (5x10^9 cfu) (*n* = 6).

<table>
<thead>
<tr>
<th>A/G ratios</th>
<th>Control dogs</th>
<th>Infected dogs</th>
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<tbody>
<tr>
<td>0 hour</td>
<td>1.07 ± 0.13</td>
<td>0.98 ± 0.12</td>
</tr>
<tr>
<td>3 hours</td>
<td>1.07 ± 0.23</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.97 ± 1.31</td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td>48 hours</td>
<td>0.95 ± 0.18</td>
<td>1.03 ± 0.50</td>
</tr>
<tr>
<td>72 hours</td>
<td>0.91 ± 0.24</td>
<td>0.88 ± 0.31</td>
</tr>
<tr>
<td>7 days</td>
<td>1.04 ± 0.19^A</td>
<td>0.66 ± 0.37^B</td>
</tr>
<tr>
<td>14 days</td>
<td>1.04 ± 0.26^A</td>
<td>0.78 ± 0.17^B</td>
</tr>
</tbody>
</table>

*A/G ratio: Albumin/globulins ratio.*

*Different superscripts* ^a,b^ *indicate significant differences* (*P* < 0.05 or more) *according to time within a given group.*

*Different superscripts* ^A,B^ *indicate significant differences* (*P* < 0.05) *between the 2 groups for a given time.*
Albumin is the most prominent of the plasma proteins and it is considered as a negative APP [4, 6, 8, 21, 25]. In animals, it constitutes between 35 and 50% of the total serum proteins, in contrast to humans and other primates in which albumin accounts for 60-67% of the total proteins [25]. In the classical literature hypoalbuminemia is very common in many illnesses and results from impairment in liver synthesis, reduced assimilation of amino-acids or increased catabolism linked to the turn off amino acids for the synthesis of other proteins (like positive acute phase proteins) in liver, or a combination of these factors [14]. The decreases in albuminemia after staphylococcal infection in the present study confirm that albumin is as a negative APP. In support of our findings, SCHREIBER et al. [25] found out decreased albuminemia in rats from 35 g/L to 23 g/L, 2 days after turpentine-induced inflammation and ORHUE et al. [23] reported a statistically significant fall in serum albumin 14 days after infection with Trypanosoma brucei in rabbits and they attributed the changes in albuminemia to both an impaired liver synthesis and increased protein loss via the gut and the kidneys. Despite the extraordinary increase in the rates of synthesis of some APPs, the rate of synthesis of serum proteins in the liver changed only moderately during inflammation. The increase in the APP synthesis causes an increase in the demand for amino-acetyl-tRNA, GTP, ATP, etc., in the liver cells, which remains however limited because of the simultaneous decrease in the synthesis rates of other proteins, and particularly of albumin, leading to a strong dispersion of the measured total protein, globulin and albumin concentrations as observed in the present study. Indeed, this protein is the most appropriate for this metabolic adaptation: its half-life is very long (T1/2: 8.2 days in dogs according to KANEKO [14]) and its total body pool is the largest among the plasma proteins. Furthermore, it has no specific function indispensable for life as indicated by the existence of apparently healthy albumin-deficient individuals in both man and rats [25]. In the same direction, CECILIANI et al. [3] consider that the down-regulation of some proteins (albumin, transferrin, transthyretin, insulin-like growth factor and others) is not related to a specific reason. It is possible to speculate on the need to divert available amino acids to the production of other APP during systemic response. The amino acids necessary for APPs synthesis derive in part from reduced synthesis of proteins that are not indispensable to the defence mechanisms such as albumin and other negative APPs, and in part from degradation of the muscle proteins via the ubiquitin-pathway [3].

In parallel to the decrease in albuminemia, slight and insignificant increases in globulins and immunoglobulins were lately evidenced on days 7 and 14, suggesting that some positive APPs would be produced mainly around 24 hours after staphylococcal inoculation. Consequently, the A/G ratios calculated for the S. intermedius infected dogs were lower than those obtained in healthy dogs, mainly on days 7 and 14 and were closed to those reported by MARTINEZ-SUBIELA et al. [21] who found albumin/globulin ratios of 0.83 and 0.42 in control dog group and in dogs with leishmaniosis, respectively. Recently, MARTINEZ-SUBIELA et al. [19-21], in accordance with our findings, reported that before therapy the dogs with leishmaniosis had an inversion of the albumin-globulin ratio, with mean value of 0.55. A slight, non-significant increase in the albumin-globulin ratio was also observed during glucocorticoid treatment [20].

<table>
<thead>
<tr>
<th>Immunoglobulin (g/L)</th>
<th>Control dogs</th>
<th>Infected dogs</th>
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<tbody>
<tr>
<td>0 hour</td>
<td>26.73 ± 1.47</td>
<td>25.25 ± 3.23</td>
</tr>
<tr>
<td>3 hours</td>
<td>26.09 ± 1.30</td>
<td>23.91 ± 3.02</td>
</tr>
<tr>
<td>24 hours</td>
<td>25.78 ± 1.11</td>
<td>22.30 ± 3.59</td>
</tr>
<tr>
<td>48 hours</td>
<td>25.55 ± 3.47</td>
<td>21.90 ± 5.60</td>
</tr>
<tr>
<td>72 hours</td>
<td>26.00 ± 2.65</td>
<td>22.44 ± 1.81</td>
</tr>
<tr>
<td>7 days</td>
<td>26.09 ± 0.74</td>
<td>29.65 ± 7.41</td>
</tr>
<tr>
<td>14 days</td>
<td>26.41 ± 3.17</td>
<td>27.65 ± 5.35</td>
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<table>
<thead>
<tr>
<th>Lysozyme (mg/L)</th>
<th>Control dogs</th>
<th>Infected dogs</th>
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<tbody>
<tr>
<td>0 hour</td>
<td>0.09 ± 0.02</td>
<td>0.60 ± 0.47</td>
</tr>
<tr>
<td>3 hours</td>
<td>0.06 ± 0.03</td>
<td>0.77 ± 0.57</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.10 ± 0.05</td>
<td>0.36 ± 0.31</td>
</tr>
<tr>
<td>48 hours</td>
<td>0.10 ± 0.01</td>
<td>1.01 ± 0.53</td>
</tr>
<tr>
<td>72 hours</td>
<td>0.10 ± 0.07</td>
<td>1.52 ± 1.13</td>
</tr>
<tr>
<td>7 days</td>
<td>0.09 ± 0.11</td>
<td>1.27 ± 0.92</td>
</tr>
<tr>
<td>14 days</td>
<td>0.06 ± 0.04</td>
<td>1.29 ± 0.98</td>
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Different superscripts $^{a,b}$ indicate significant differences (P < 0.05 or more) according to time within a give group. Different superscripts $^{A,B}$ indicate significant differences (P < 0.05 or more) between the 2 groups for a given time.

TABLE II: Variations of the plasma immunoglobulin concentrations and lysozyme activities according to time in control healthy dogs (n = 6) and in dogs subcutaneously inoculated with a Staphylococcus intermedius suspension (5x10$^9$ cfu) (n = 6). Results are expressed as means ± standard deviation (SD).
Furthermore, these authors observed a strong increase in γ-globulin concentrations (28.2 g/L) in naturally diseased dogs compared to the controls (11.5 g/L) [21]. BURTIS [2] also stated that increased concentrations of immunoglobulins are seen in both acute and chronic infections. Measurement of a particular immunoglobulin class, such as IgM, brings no diagnostic value whereas the evidence of an elevated immunoglobulin concentration at least provides an important aid to the diagnosis of many infectious diseases [13]. In the present study the zinc sulphate turbidity test was used to evaluate the total amount of immunoglobulins in serum samples. Total immunoglobulins include natural antibodies, which are genetically determined and are produced as a result of immune maturation of organism, asymptomatic infections or the existence of antigens which are common for different organisms. Total immunoglobulins include also antibodies against Staphylococcus intermedius, produced in the course of the experimental infection. Immunoglobulin classes (IgM, IgG, IgA and IgE) are not identified by this method. In dogs with experimental staphylococcal infection the increase of immunoglobulins is not statistically significant as compared to control animals, indicating that the level of resistance due to immunoglobulins is not highly efficient. To analyse the biological effects of immunoglobulins, a longer period of time is needed, because the amount of synthesized immunoglobulins in such infections increases after 21st day. In our study we used dynamics including evaluation up to the 14th day.

The role of lysozyme in host defence against infection remains elusive [5], but its elevation as part of non specific humoral immunity in rabbits following E.coli infection, showed its involvement in response to the administered antigen [26, 29]. In the same way, it was demonstrated that the circulating lysozyme activity play an important role in the natural immunity against coccidiosis in broiler chickens [15, 27]. LEITCH et al. [17] and PAWLIKOWSKA and DEPTULA [24] found that alterations of the lysozyme activity manifested earlier than positive titres of the specific antibodies developed. Consequently, the innate immunity may be established very early and may participate to the defence of dogs against infections diseases. Corroborating that, SOTIROV (personal communication, unpublished data) has demonstrated that the serum lysozyme activity was independent of the age of the dogs. In agreement with that, increased serum lysozyme activities were evidenced in Staphylococcus infected dogs compared to the healthy dogs since 48 hours after bacterial inoculation and these changes were maximal on day 3 and prolonged until the 14th day. In addition, LEITH and WILLCOX [28] have demonstrated the specific anti-staphylococcal actions of lactoferrin and lysozyme.

As a conclusion, it was observed an overall increase in γ-globulin concentrations (28.2 g/L) in naturally diseased dogs compared to the controls (11.5 g/L) [21]. BURTIS [2] also stated that increased concentrations of immunoglobulins are seen in both acute and chronic infections. Measurement of a particular immunoglobulin class, such as IgM, brings no diagnostic value whereas the evidence of an elevated immunoglobulin concentration at least provides an important aid to the diagnosis of many infectious diseases [13]. In the present study the zinc sulphate turbidity test was used to evaluate the total amount of immunoglobulins in serum samples. Total immunoglobulins include natural antibodies, which are genetically determined and are produced as a result of immune maturation of organism, asymptomatic infections or the existence of antigens which are common for different organisms. Total immunoglobulins include also antibodies against Staphylococcus intermedius, produced in the course of the experimental infection. Immunoglobulin classes (IgM, IgG, IgA and IgE) are not identified by this method. In dogs with experimental staphylococcal infection the increase of immunoglobulins is not statistically significant as compared to control animals, indicating that the level of resistance due to immunoglobulins is not highly efficient. To analyse the biological effects of immunoglobulins, a longer period of time is needed, because the amount of synthesized immunoglobulins in such infections increases after 21st day. In our study we used dynamics including evaluation up to the 14th day.

The role of lysozyme in host defence against infection remains elusive [5], but its elevation as part of non specific humoral immunity in rabbits following E.coli infection, showed its involvement in response to the administered antigen [26, 27]. Notably, the lysozyme activity were dramatically altered (this parameter has increased two fold in the experimental group) whereas the changes in globulins and immunoglobulins were much lower, leading to consider the serum lysozyme activity as a more sensitive marker of inflammation / infection in dogs than albuminemia, or the A/G ratio.

References


